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# Cardiac Growth Factors in Human Hypertrophy

## Relations With Myocardial Contractility and Wall Stress

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**Abstract**—The aim of the present study was to investigate whether and which cardiac growth factors are involved in human hypertrophy, whether growth factor synthesis is influenced by overload type and/or by the adequacy of the hypertrophy, and the relationships between cardiac growth factor formation and ventricular function. Cardiac growth factor formation was assessed by measuring aorta-coronary sinus concentration gradient in patients with isolated aortic stenosis (n=26) or regurgitation (n=15) and controls (n=12). Gene expression and cellular localization was investigated in ventricular biopsies using reverse transcriptase–polymerase chain reaction and in situ hybridization. Cardiac hypertrophy with end-systolic wall stress  $<90$  kdyne/cm<sup>2</sup> was associated with a selective increased formation of insulin-like growth factor (IGF)-I in aortic regurgitation and of IGF-I and endothelin (ET)-1 in aortic stenosis. mRNA levels for IGF-I and preproET-1 were elevated and mainly expressed in cardiomyocytes. At stepwise analysis, IGF-I formation was correlated to the mean velocity of circumferential fiber shortening ( $r=0.86$ ,  $P<0.001$ ) and ET-1 formation to relative wall thickness ( $r=0.82$ ,  $P<0.001$ ). When end-systolic wall stress was  $>90$  kdyne/cm<sup>2</sup>, IGF-I and ET-1 synthesis by cardiomyocytes was no longer detectable, and only angiotensin (Ang) II was generated, regardless of the type of overload. The mRNA level for angiotensinogen was high, and the mRNA was exclusively expressed in the interstitial cells. Ang II formation was positively correlated to end-systolic stress ( $r=0.89$ ,  $P<0.001$ ) and end-diastolic stress ( $r=0.84$ ,  $P<0.001$ ). Multivariate stepwise analysis selected end-systolic stress as the most predictive variable and left ventricular end-diastolic pressure as the independent variable for Ang II formation ( $r=0.93$ ,  $P<0.001$ ). In conclusion, the present results indicate that the course of human left ventricular hypertrophy is characterized by the participation of different cardiac growth factors that are selectively related both to the type of hemodynamic overload and to ventricular function. (*Circ Res.* 1999;85:57-67.)

**Key Words:** myocardial hypertrophy ■ aortic valve disease ■ endothelin-1  
■ insulin-like growth factor-I ■ angiotensin II

A number of studies have investigated the mechanisms involved in the development of myocardial hypertrophy (for review, see References 1 through 4). Evidence has been presented that hemodynamic overload is the initial stimulus and that hypertrophy results from the interaction between mechanical forces and neuroendocrine factors, leading to an increased cardiac protein synthesis according to a recapitulation of a fetal pattern of gene expression, with upregulation of some genes and downregulation of others.<sup>2,3</sup> Studies performed in animal models of hypertrophy and cardiac myocytes cultured from neonatal rat hearts in vitro have shown that a number of factors, including norepinephrine, angiotensin (Ang), endothelin (ET), insulin-like growth factor (IGF)-I, interleukin-1 $\beta$ , and tumor necrosis factor- $\alpha$  (see Reference 5), are involved in,

or are potential stimuli for, myocyte hypertrophy. The results obtained from cultured myocytes and the experimental models of hypertrophy cannot, however, be easily extrapolated to human hypertrophy because of species and developmental stage differences and the large variety in the duration, extent, observation moment, and types of hypertrophy. Although studies performed in patients before and after valve replacement have confirmed that in humans as well as in animals hemodynamic overload plays a key role in the occurrence of myocardial hypertrophy,<sup>6-8</sup> only very little information is available regarding the cardiac growth factors involved in human hypertrophy. An increased expression of mRNA for transforming growth factor- $\beta_1$  and IGF-I have been reported in idiopathic hypertrophic cardiomyopathy and in aortic valve stenosis (AS).<sup>9</sup>

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**TABLE 1. Main Echocardiographic and Hemodynamic Characteristics of Subjects Investigated**

	Aortic Stenosis			Aortic Regurgitation		Mitral Stenosis (n=6)
	Controls (n=12)	Adequate (n=17)	Inadequate (n=9)	Adequate (n=7)	Inadequate (n=8)	
Age, y	57±7	63±10	63±9	55±6	62±7	63±8
Aortic gradient, mm Hg	...	73±15	73±17	2±4	5±7	...
Regurgitant fraction, %	...	...	...	54±6	52±7	...
Aortic valve area, cm <sup>2</sup>	...	0.8±0.2	0.7±0.1	...	...	...
Mitral valve area, cm <sup>2</sup>	...	3.9±0.3	3.8±0.4	3.4±0.9	4.3±0.2	1.0±0.1
Left ventricular mass, g/m <sup>2</sup>	110±14	189±26*	189±33*	150±4*	205±53†‡	113±14
Relative wall thickness, %	36±3	54±7*	44±5†§	35±3	36±4	43±3†
LVIDDI, cm/m <sup>2</sup>	2.7±0.1	2.9±0.3	3.0±0.3	3.2±0.1*	3.5±0.5	2.8±0.2
Vcf, s <sup>-1</sup>	1.15±0.18	1.16±0.14	0.87±0.10†¶	1.12±0.16	0.56±0.25*§	...
E/A ratio	1.24±0.17	1.05±0.16	0.92±0.11†	...	...	...
End-systolic stress, kdyne/cm <sup>2</sup>	70±6	69±12	109±14*¶	82±4*	115±21†‡	64±11
End-diastolic stress, kdyne/cm <sup>2</sup>	14±3	19±4†	35±6*¶	30±7†	49±16†‡	11±8
LVESVI, mL/m <sup>2</sup>	32±4	31±7	44±9  ‡	38±2†	67±19†‡	36±6
LVEDVI, mL/m <sup>2</sup>	86±9	78±16	99±10  §	109±22	138±19*‡	79±2
Ejection fraction, %	63±4	59±10	55±10	63±8	52±9  ‡	55±6
LVEDP, mm Hg	8±1	19±3*	29±3*¶	18±5*	30±6†§	9±6
Vcf/LVEDVI	0.014±0.003	0.015±0.004	0.009±0.002†¶	0.011±0.002	0.004±0.002*¶	...
Vcf/end-systolic stress	0.017±0.004	0.018±0.004	0.008±0.001*¶	0.014±0.002	0.005±0.003*¶	...

LVIDDI indicates left ventricular internal diastolic dimension index; LVESVI, left ventricular end-systolic volume index.

||*P*<0.05, †*P*<0.01, and \**P*<0.001 vs controls at multiple comparison test; ‡*P*<0.05, §*P*<0.01, and ¶*P*<0.001 vs adequate hypertrophy at multiple comparison test.

The present study was therefore planned to investigate the following: (1) whether cardiac growth factors, specifically Ang II, ET-1, and IGF-I, which have been found to be more frequently operating in experimental studies, are also involved in human hypertrophy due to AS or aortic regurgitation (AR); (2) whether gene program synthesis of these growth factors is modified during the transition from compensated (or adequate), normalizing wall stress, to decompensated (or inadequate) hypertrophy, with elevated wall stress; and (3) the relationship between cardiac growth factor formation and left ventricular function.

## Materials and Methods

### Subjects Investigated

We investigated 41 patients with aortic valve disease (AS, n=26; AR, n=15) and left ventricular hypertrophy with stable hemodynamic status and without any clinical signs of heart failure. Patients with AS were enrolled if aortic valve area (AVA) was <1 cm<sup>2</sup>, in the absence of significant AR. Patients with AR were enrolled when the regurgitant fraction (RF) was ≥40% and the valve gradient was <20 mm Hg.

The control group was made up of 12 normotensive patients who underwent coronary angiography for atypical chest pain. Angiography and routine diagnostic procedures did not reveal any abnormalities. Six patients with mitral stenosis (4 with atrial fibrillation and increased pulmonary pressure) were also investigated as diseased controls. All subjects were studied after a week of a normal sodium diet. Diuretics and/or angiotensin-converting enzyme inhibitors were withheld a week before the study. The characteristics of patients and controls are reported in Table 1.

Eighteen patients with AS, 11 with AR, and 4 with mitral stenosis underwent surgical valve replacement, and myocardial ventricular

biopsies were collected from those patients who gave written informed consent (9 with AS, 6 with AR, and 4 with mitral stenosis). Cardiac specimens were also obtained from the explanted hearts of 5 donors (age 44±6 years) with no history of cardiac disease who had been excluded from organ donation for noncardiac reasons (control hearts).

The protocol of this study complies with the principles of the Helsinki declaration,<sup>10</sup> and all patients gave their informed consent to participate in the study and to have myocardial biopsies performed and ventricular specimens used for experimentation. All subjects underwent a complete clinical and instrumental evaluation for diagnostic purposes. Coronary artery disease (defined as 50% or more luminal diameter narrowing of at least 1 major coronary artery at angiography) was present in 8 patients with AS and 3 with AR, with 5 and 1, respectively, suffering from stable effort angina. Patients were excluded if they had development or worsening of aortic valve disease within the previous 3 months; diastolic blood pressure >90 mm Hg; a recent history (<6 months) of effort angina, angina at rest, or myocardial infarction; or echocardiographic evidence of additional valve or congenital heart disease.

The capacity of myocardial hypertrophy for keeping pace with hemodynamic overload was assessed on the basis of meridional end-systolic wall stress (ESS). Patients were considered to have adequate hypertrophy when ESS values were <90 kdyne/cm<sup>2</sup> and to have inadequate hypertrophy when ESS was >90 kdyne/cm<sup>2</sup>.<sup>11,12</sup>

### Echocardiographic and Hemodynamic Measurements

All echocardiographic examinations were performed according to the American Society of Echocardiography.<sup>13</sup> All measurements were performed prospectively. Left ventricular hypertrophy was considered to be present if the left ventricular mass, calculated according to the Devereux formula<sup>14</sup> and indexed for body surface area, was ≥134 g/m<sup>2</sup> for men and 110 g/m<sup>2</sup> for women.<sup>15</sup>

Relative wall thickness (RWT) was calculated according to the following formula:  $RWT = (2 \times PWT) / LVDD$ , where  $PWT$  is posterior wall thickness and  $LVDD$  is left ventricular internal diastolic dimension.

The degree of AS was assessed by estimation of AVA, which was based on the principle of continuity of flow. Specifically, the cross-sectional area (CSA) at the left ventricular outflow tract ( $CSA_{LVOT}$ ) was measured with 2-dimensional echocardiography, and the velocity-time integrals in the left ventricular outflow tract ( $V_{LVOT}$ ) and in the AS jet ( $V_{AO}$ ) were measured with Doppler echocardiography, so that AVA was calculated according to the following formula:  $AVA = CSA_{LVOT} \times (V_{LVOT} / V_{AO})$ .

The maximum transaortic pressure gradient (APG) was calculated from the maximum aortic jet velocity ( $V_{max}$ ) using the Bernoulli equation, as follows:  $APG = 4 \times (V_{max})^2$ .

AR was assessed by estimation of the RF. Regurgitant stroke volume ( $SV_{REG}$ ) was calculated as the difference between total stroke volume ( $SV_{TOT}$ ) (calculated as the cross-sectional area of flow times the velocity-time integral of transvalvular flow across the regurgitant valve) and forward stroke volume ( $SV_{FOR}$ ) (calculated as antegrade flow across a different and nonregurgitant valve). RF was then calculated as  $SV_{REG} / SV_{TOT}$ .

Mitral stenosis was diagnosed when mitral valve area, measured by either the pressure half-time method or direct planimetry, was  $<1.5 \text{ cm}^2$ , with no or only mild mitral regurgitation at continuous-wave Doppler echocardiography. Left ventricular systolic function was evaluated by measuring the ejection fraction and the mean midwall velocity of circumferential fiber shortening (Vcf), using Doppler and 2-dimensional echocardiographic data.

The Vcf was calculated as  $Vcf = FS / ET_s$ , where the fractional shortening (FS) is  $[(LVDD - \text{left ventricular internal systolic dimension}) / LVDD] \times 100$  and the ejection time (ETs) was derived from the duration of aortic valve opening. Values of Vcf were normalized by left ventricular end-diastolic volume index (LVEDVI) and ESS to evaluate cardiac contractility independently of both preload (LVEDVI) and end-systolic wall tension (ESS).

Diastolic relaxation was assessed by Doppler evaluation of the early maximum (E) and atrial (A) left ventricular filling inflow velocities and their ratio (E/A). Estimates of left ventricular meridional wall stress (WS) were made from M-mode data in combination with pressure data, using the following formula:  $WS = 0.334 \times P \times LVID / [PWT \times (1 + PWT / LVID)]$ , where  $P$  is the left ventricular pressure and  $LVID$  is the left ventricular internal dimension.

ESS was calculated using systolic blood pressure according to Reichel et al.<sup>12</sup> End-diastolic meridional wall stress (EDS) was calculated using invasive measurements of left ventricular pressure taken after the a wave.<sup>16</sup>

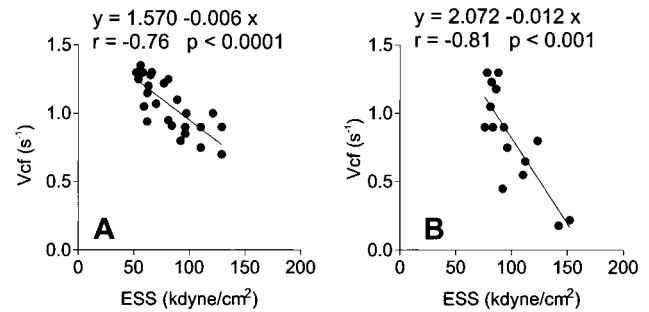
Echocardiographic measurements were read independently by 2 observers unaware of patient identity and of the radioimmunological assays of cardiac growth factors. Interobserver and intraobserver variability were  $4.1 \pm 0.5\%$  and  $2.5 \pm 0.3\%$  for cavity size and  $3.7 \pm 0.4\%$  and  $2.1 \pm 0.3\%$  for wall thickness, respectively.

## Estimation of the Cardiac Production of Growth Factor Peptides

Patients were premedicated with oral diazepam (10 mg) 1 hour before the study. After 20 minutes of supine rest and 10 minutes after catheter positioning, coronary blood flow was measured twice at 5-minute intervals with the thermodilution technique,<sup>17</sup> and blood samples for the determination of cardiac oxygen extraction (arterial-coronary sinus difference) were obtained. Blood samples (10 mL) for plasma renin activity (PRA) and growth factors assays were contemporaneously drawn from the aorta, coronary sinus, and antecubital vein according to procedures previously described.<sup>17-19</sup>

### ET-1, Big ET, and IGF-I Assays

Cardiac formation of ET-1, Big ET, and IGF-I was expressed as the aorta-coronary sinus concentration gradient indexed by coronary flow and cardiac mass. Plasma extraction and radioimmunoassay of ET-1 were performed as previously described.<sup>19</sup> The coefficients of



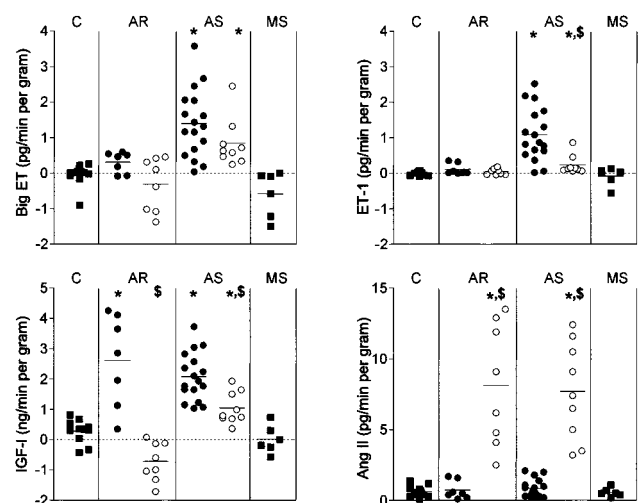
**Figure 1.** Correlation between mean midwall Vcf and ESS in patients with AS (A) and AR (B).

intra-assay and interassay variations were 4% and 10%, respectively. Big ET was assayed in extracted samples, as for ET-1, by using a specific rabbit polyclonal antibody (Peninsula Laboratories, Inc). Intra- and interassay variabilities were 2.8% and 9.7%, respectively.

IGF-I was extracted from acidified plasma samples using disposable chromatographic cartridges (Waters Associated), previously activated with 60% acetonitrile in 1% trifluoroacetic acid (TFA) in distilled water (1 mL, once), followed by 1% TFA in distilled water (3 mL, 3 times). After loading, the column was washed twice with 1 mL of 1% TFA, and the adsorbed peptide was eluted with 3 mL of 60% acetonitrile in 1% TFA. Eluates were dried and stored at  $-80^\circ\text{C}$ . IGF-I was measured with radioimmunoassay using a specific rabbit polyclonal antibody (Peninsula Laboratories, Inc). The IGF-I recovery rate was  $95 \pm 2\%$ . Intra- and interassay variabilities were 3.5% and 10.3%, respectively. The minimum detectable concentration was 1 ng/mL.

### Ang I, Ang II, and PRA

Ang I and Ang II cardiac formation was measured by the study of  $^{125}\text{I}$ -labeled Ang I kinetics, which provides precise information about the amount of Ang I de novo formed by a tissue and the total amount of Ang II resulting both from the conversion of Ang I (arterially delivered) and from Ang II formed by the tissue.<sup>20,21</sup>  $^{125}\text{I}$ -labeled Ang I and  $^{125}\text{I}$ -labeled Ang II extraction, the fractional conversion rate of Ang I to Ang II, and cardiac de novo Ang I and Ang II production



**Figure 2.** Big ET, ET-1, IGF-I, and Ang II cardiac formation in patients with AS, AR, and mitral stenosis (MS). ■ indicates healthy controls (C) and controls with MS; ●, patients with adequate hypertrophy; and ○, patients with inadequate hypertrophy, both for AS and for AR. Statistical significance in growth factor cardiac generation in comparison with control and adequate hypertrophy is reported at the top of columns (\* $P < 0.05$  vs control, \$ $P < 0.05$  vs adequate hypertrophy at multiple comparison test).

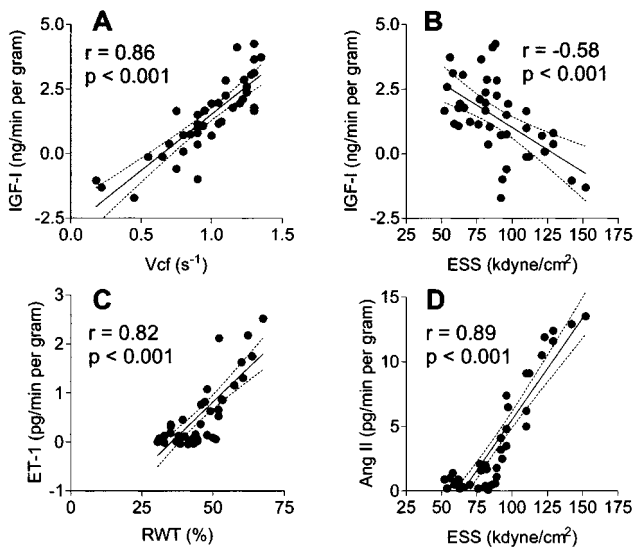
**TABLE 2. Univariate and Stepwise Multiple Regression Among Ang II, ET-1, and IGF-I Cardiac Production and Hemodynamic Parameters**

	All Patients (n=41)		Aortic Regurgitation (n=15)		Aortic Stenosis (n=26)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
IGF-I						
ESS	-0.58*	0.001	-0.59	0.05	-0.49	0.05
AVG	0.28	...	...	...	0.06	...
EDS	-0.53	0.001	-0.43	...	-0.48	0.05
LVMi	-0.29	...	-0.46	...	0.03	...
RWT	0.40	0.01	-0.12	...	0.68	0.001
LVEDVI	-0.47	0.01	-0.30	...	-0.49	0.05
LVESVI	-0.63	0.001	-0.67	0.01	-0.30	...
EF	0.33	0.05	0.72	0.01	-0.12	...
LVEDP	-0.45	0.01	-0.49	...	-0.22	...
Vcf	0.86*	0.001	0.87*	0.001	0.76*	0.001
E/A ratio	...	...	...	...	0.32	...
Multiple regression						
Vcf+ESS	0.89	0.001				
ET-1						
ESS	-0.60	0.001	-0.03	...	-0.64	0.001
AVG	0.58	0.001	...	...	0.16	...
EDS	-0.59	0.001	0.06	...	-0.71	0.001
LVMi	-0.07	...	0.12	...	-0.29	...
RWT	0.82*	0.001	-0.28	...	0.82*	0.001
LVEDVI	-0.55	0.001	-0.11	...	-0.38	...
LVESVI	-0.41	0.01	-0.12	...	-0.31	...
EF	-0.02	...	0.10	...	-0.02	...
LVEDP	-0.39	0.05	0.16	...	-0.51	0.01
Vcf	0.52	0.001	0.02	...	0.68	0.001
E/A ratio	...	...	...	...	0.63*	0.001
Multiple regression						
RWT+E/A ratio					0.87	0.001
Ang II						
ESS	0.89*	0.001	0.96*	0.001	0.85*	0.001
AVG	-0.16	...	...	...	0.11	...
EDS	0.84	0.001	0.91	0.001	0.84	0.001
LVMi	0.45	0.01	0.92	0.001	-0.31	...
RWT	-0.44	0.01	-0.36	...	-0.49	0.05
LVEDVI	0.61	0.001	0.76	0.001	0.42	0.05
LVESVI	0.80	0.001	0.86	0.001	0.71	0.001
EF	-0.49	0.01	-0.58	0.05	-0.48	0.05
LVEDP	0.88*	0.001	0.89	0.001	0.84*	0.001
Vcf	-0.78	0.001	-0.79	0.001	-0.74	0.001
E/A ratio	...	...	...	...	-0.20	...
Multiple regression						
ESS+LVEDP	0.93	0.001			0.90	0.001

AVG indicates aortic valve gradient; LVMi, left ventricular mass index; LVESVI, left ventricular end-systolic volume index; and EF, ejection fraction.

\*Independent variable at stepwise regression.





**Figure 3.** Correlation with IGF-I cardiac formation and Vcf (A) and ESS (B), between ET-1 cardiac formation and RWT (C), and between Ang II cardiac formation and ESS (D) in all patients with AS and AR considered as a whole group.

were used for the evaluation of cardiac  $^{125}\text{I}$ -labeled Ang I and  $^{125}\text{I}$ -labeled Ang II kinetics. The validity and reliability of these parameters were confirmed in previous studies.<sup>20–22</sup> To calculate the amount of Ang II formed by PRA during blood transcatheter passage, we determined the mean transcoronary transit time according to Gorlin and Storaasli<sup>23</sup> in a preliminary study. Ang I and Ang II concentrations in plasma were measured with radioimmunoassay, using specific polyclonal antibodies (Peninsula Laboratories, Inc, for Ang I, and ITS Technogenetic for Ang II), as previously described in detail.<sup>18</sup> Overall intra- and interassay variation coefficients were

6.3% and 12.4% for Ang I and 7.7% and 13.6% for Ang II, respectively. PRA measurement was performed with a commercial kit (Sorin Biomedica).

### Quantification of Growth Factor mRNA Levels in the Myocardium

Transmural myocardial biopsies (10 to 20 mg) were obtained from approximately the same region of the left ventricular free wall. Biopsy specimens were immediately put in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until processing. Myocardial levels of preproET-1 (ppET-1), angiotensinogen (AGTN), and IGF-I transcripts were quantified with reverse transcriptase–polymerase chain reaction (RT-PCR) using GAPDH as internal standard, according to Li et al.<sup>9</sup>

Total mRNA was isolated from homogenized frozen samples using TRIzol reagent (GIBCO-BRL/Life Technologies) as outlined by the manufacturer and reverse transcribed using oligo(dT).<sup>20</sup> PCR primers were designed according to Li et al<sup>9</sup> for GAPDH and IGF-I, according to Pagotto et al<sup>24</sup> for ppET-1 and according to Paul et al<sup>25</sup> for AGTN. All primers were purchased from Pharmacia. To ensure that different amounts of PCRs on myocardial biopsies were not due to markedly different starting concentrations of mRNA, PCR analysis was performed for the internal control mRNA (GAPDH) on serial 2-fold dilutions of cDNA for each sample. The last dilution giving a positive reaction for GAPDH was used to equalize the amount of cDNA used in each PCR.

PCR reactions were performed according to Li et al<sup>9</sup> in a DNA thermal cycler (Perkin Elmer Cetus). GAPDH densities and the growth factor bands were analyzed using a computer image densitometer (Qwin, Leica). The ratio of the growth factor to GAPDH was determined.

### Localization of Growth Factor mRNA in the Myocardium

The in situ hybridization procedure was performed as previously described<sup>26</sup> using cDNA photobiotin-labeled (Vector Laboratories) probes for ppET-1 (ET1c, American Type Culture Collection [ATCC] No. 65698), AGTN (ATCC No. 822996), IGF-I (ATCC No. 59944), and GAPDH (pHcGAP, ATCC No. 57090). Negative controls were performed by testing the sections with hybridization mixture (1) without the probe, (2) after incubation with RNase A (0.05 mg/mL=4.7 Kunitz units/mL) for 1 hour at  $37^{\circ}\text{C}$ , and (3) with application of inappropriate probe (plasmid vector pBR322). Positive controls were obtained for each sample using a cDNA probe for the housekeeping gene GAPDH. Myocytes were stained using a specific anti-human myosin antibody (M8421, Sigma) and a secondary fluorescein-conjugated antibody (F4143, Sigma). Each stained histological section was examined under a microscope (DMRB, Leica) connected to a computerized image-analysis system (Qwin, Leica).

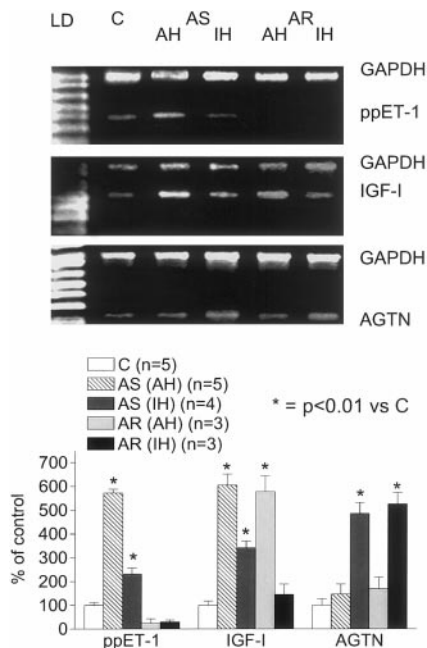
### Statistical Analysis

Data are expressed as mean $\pm$ SD. Comparison between groups was performed using a 1-way ANOVA and Student *t* test followed by the Tukey multiple-range comparison test, as appropriate. Univariate linear relations were analyzed with the Pearson correlation. A stepwise multiple regression analysis was used for multivariate re-evaluation of univariate correlations. Variables included in the stepwise regression analysis were the following: ESS, aortic valve gradient, EDS, left ventricular mass index, LVEDVI, left ventricular end-systolic volume index, RWT, left ventricular end-diastolic pressure (LVEDP), ejection fraction, Vcf, and E/A ratio. The significance level for univariate and multivariate testing was set at 0.05. All calculations were performed using BMDP statistical software.

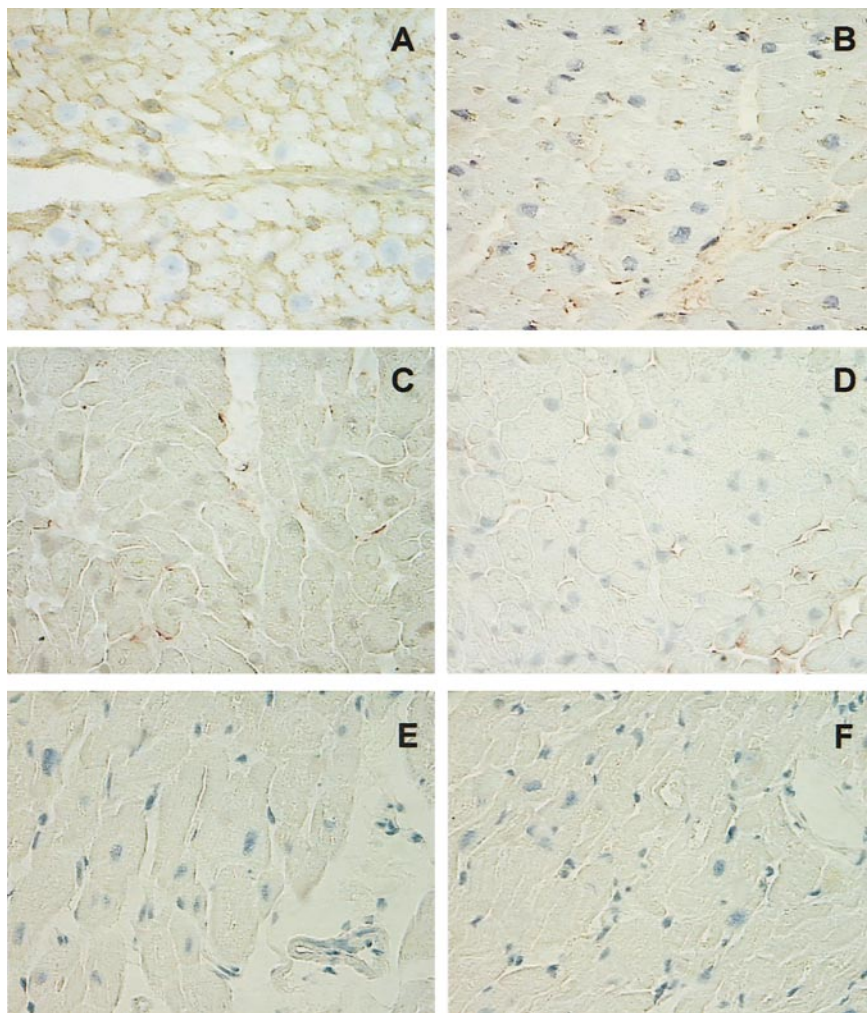
## Results

### Echocardiographic and Hemodynamic Characteristics of the Patients

The echocardiographic and hemodynamic characteristics of the patients are reported in Table 1. The myocardial contrac-



**Figure 4.** Top, RT-PCR products of ppET-1, IGF-I, and AGTN from the myocardium of the hearts of an organ donor (C) and from patients with AS or AR with adequate (AH) or inadequate (IH) hypertrophy. LD indicates DNA molecular size markers. At bottom, the densitometric growth factor/GAPDH ratio is expressed as a percentage of values obtained in control hearts. Data are presented as mean $\pm$ SD.



**Figure 5.** In situ hybridization for GAPDH (A), IGF-I (B), ppET-1 (C), and AGTN (D) in myocardial biopsies taken from the heart of an organ donor excluded from cardiac donation because of the mismatch of cardiac size with the chest of recipient. E and F, Negative controls obtained after incubation with RNase A (E) and with plasmid vector pBR322 (F). Magnification,  $\times 400$ .

tile status (as expressed by the ratios Vcf/LVEDVI and Vcf/ESS) of all patients except those with adequate hypertrophy (ie, with ESS  $<90$  kdyne/cm<sup>2</sup>) was significantly reduced in comparison with controls. Left ventricular rate-corrected Vcf was linearly and inversely related to ESS in patients both with AS and AR, but the slopes of the 2 equations indicated an earlier depression of systolic function at increasing ESS values in patients with AR than in those with AS (Figure 1).

### Plasma Assays of Cardiac Formation of Growth Factors

#### IGF-I

In the AR group, cardiac formation of IGF-I was significantly increased only in patients with ESS  $<90$  kdyne/cm<sup>2</sup> (Figure 2). Both groups of patients with AS showed a higher cardiac formation of IGF-I than controls, with higher values in patients with ESS  $<90$  kdyne/cm<sup>2</sup> than in those with ESS  $>90$  kdyne/cm<sup>2</sup> ( $P<0.01$ ) (Figure 2). IGF-I formation was positively related to Vcf at both univariate and stepwise regression analysis in patients with AR ( $r=0.87$ ,  $P<0.001$ ) and in patients with AS ( $r=0.76$ ,  $P<0.001$ ) (Table 2). At stepwise analysis with all patients considered as a whole group, Vcf and ESS were the 2

variables that were independently related to IGF-I formation (Table 2, Figure 3A and 3B).

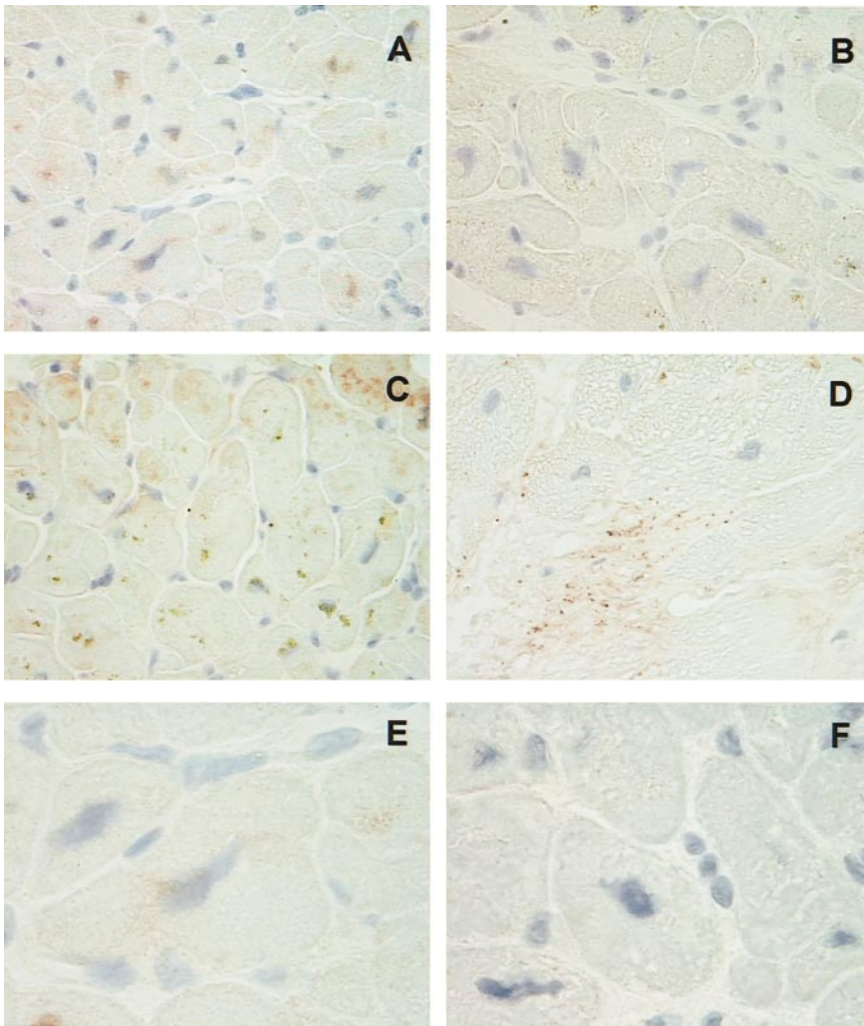
#### ET-1 and Big ET

Only AS patients had significantly higher cardiac formation of ET-1 and Big ET than controls, with patients with adequate hypertrophy showing higher values of ET than patients with inadequate hypertrophy ( $P<0.01$ ) (Figure 2). At multivariate stepwise analysis, RWT was the only variable independently related to ET-1 formation both in the AS patients and in the whole group (Table 2, Figure 3C). ET-1 formation was not significantly different in patients with or without effort stable angina ( $1.47\pm0.77$  versus  $0.88\pm0.66$  pg/min per gram,  $P=0.12$ ).

#### Ang I and Ang II

PRA in controls was  $0.77\pm0.17$  ng/mL per hour, and no significant differences were found among the various groups of patients ( $F=0.76$ ). The aorta-coronary sinus gradient of Ang I and II in controls and in patients with mitral stenosis was  $\approx 0$ . However, the kinetic study of <sup>125</sup>I-labeled Ang I showed that the de novo formation of Ang I and Ang II on average balanced out the extraction of Ang I (degradation and conversion of <sup>125</sup>I-labeled Ang I to <sup>125</sup>I-labeled Ang II) and the degradation of Ang II during blood transcardiac passage.





**Figure 6.** In situ hybridization for IGF-I mRNA in myocardial biopsies from patients with AR (A, B, and E) or AS (C and D) with adequate (A, C, and E) or inadequate (B and D) hypertrophy. F, RNase treatment of the same specimen of panel E. Magnification:  $\times 400$  (A through D) and  $\times 1000$  (E and F). A and E, Positive mRNA signal in cardiomyocytes of a biopsy taken from a patient with AR and adequate hypertrophy. B, Negative mRNA expression in AR with inadequate hypertrophy. C, Positive signal of mRNA in cardiomyocytes of a patient with AS and adequate hypertrophy. D, Positive signal in interstitial cells of a patient with AS and inadequate hypertrophy.

In all of the patients with  $ESS < 90$  kdyne/cm<sup>2</sup>, cardiac Ang formation did not differ from that of controls (Figure 2). Conversely, in all of the patients with  $ESS > 90$  kdyne/cm<sup>2</sup>, regardless of the type of aortic valve disease, cardiac Ang I formation and conversion of Ang I to Ang II were notably increased, thus resulting in augmented Ang II formation (Figure 2). Ang II formation was positively correlated to ESS ( $r=0.89$ ,  $P<0.001$ ), EDS ( $r=0.84$ ,  $P<0.001$ ), and LVEDP ( $r=0.88$ ,  $P<0.001$ ) and negatively to Vcf ( $r=-0.78$ ,  $P<0.001$ ) (Table 2). When multivariate stepwise analysis was performed, ESS continued to be the most predictive independent variable for Ang II formation (Table 2) (Figure 3D). The addition of LVEDP to ESS significantly improved the correlation (ESS and LVEDP,  $r=0.93$ ,  $P<0.001$ ) (Table 2).

#### RT-PCR Assay of Cardiac Formation of Growth Factors

RT-PCR data (densitometric ratio of growth factor/GAPDH) showed that the expression of mRNA for IGF-I in AR hearts was significantly increased only in adequate hypertrophy ( $1.21 \pm 0.14$  [+478% versus control;  $P<0.005$ ] in adequate hypertrophy and  $0.31 \pm 0.09$  [+46% versus control; NS] in inadequate hypertrophy), whereas in AS it was increased in

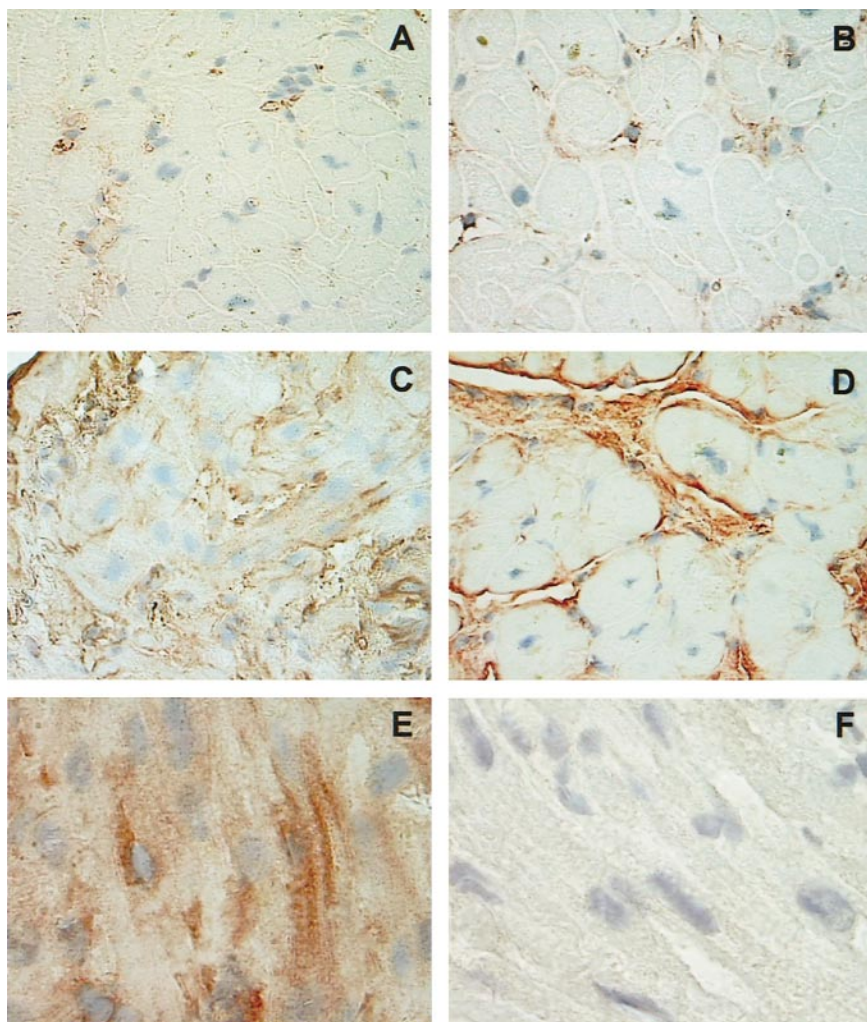
both groups ( $1.27 \pm 0.1$  [+505% versus control] in adequate hypertrophy and  $0.72 \pm 0.06$  [+242% versus control] in inadequate hypertrophy;  $P<0.0001$  for both) (Figure 4). The expression of mRNA for ET-1 was increased only in AS, especially in adequate hypertrophy ( $0.64 \pm 0.02$  [+472% versus control;  $P<0.0001$ ] in adequate hypertrophy and  $0.26 \pm 0.03$  [+132% versus control;  $P<0.001$ ] in inadequate hypertrophy) (Figure 4). mRNA for AGTN was overexpressed in patients with increased ESS with both AR and AS ( $0.35 \pm 0.03$  [+386% versus control;  $P<0.0001$ ] and  $0.38 \pm 0.04$  [+426% versus control;  $P<0.002$ ], respectively) (Figure 4).

#### Hybridization Studies

Negative and positive controls for hybridization showed that it was specific for mRNA and that the mRNA in the biopsies was intact (Figures 5 through 8). In the hearts of both healthy donors and patients with mitral stenosis, mRNA for IGF-I, ppET-1, and AGTN was expressed only in trace amounts (Figure 5).

In the specimens from patients with AR who had adequate hypertrophy, mRNA for IGF-I (Figure 6) was clearly expressed in myocytes and only mildly in the interstitial cells. No expression of mRNA for ppET-1 (Figure 7) and AGTN





**Figure 7.** In situ hybridization for ppET-1 mRNA in myocardial biopsies from patients with AR (A and B) or AS (C through E) with adequate (A, C, and E) or inadequate (B and D) hypertrophy. F, RNase treatment of the same specimen as in panel E. Magnification:  $\times 400$  (A through D) and  $\times 1000$  (E and F). A, mRNA signal for ppET-1 is detectable only in endothelial cells in a myocardial specimen from a patient with AR and adequate hypertrophy. B, Pale signal of mRNA expression in interstitial cells in AR with inadequate hypertrophy. C and E, Markedly positive signal of mRNA in cardiomyocytes of a patient with AS and adequate hypertrophy. D, Markedly positive signal in interstitial cells of a patient with AS and inadequate hypertrophy.

(Figure 8) was detectable in the cardiomyocytes of this group of patients. In AS and adequate hypertrophy, mRNA for ppET-1 was markedly expressed in cardiomyocytes and to a lesser extent also in vascular wall and interstitial cells (Figure 7C and 7E). In this group of patients, there was an augmented expression of mRNA for IGF-I in myocytes (Figure 6C).

In patients with AS and inadequate hypertrophy, the mRNA expression of ppET-1 was almost absent in myocytes, whereas it was evident in interstitial cells (Figure 7D). mRNA for IGF-I was undetectable in myocytes and only mildly expressed by interstitial cells (Figure 6D).

In patients with  $ESS > 90$  kdyne/cm<sup>2</sup>, mRNA expression for AGTN was notably enhanced in the interstitial cells in both AR (Figure 8B and 8E) and AS (Figure 8D). In patients with  $ESS < 90$  kdyne/cm<sup>2</sup>, regardless of the type of valve defect, mRNA expression for AGTN was very weak or absent (Figure 8A and 8C), thus confirming that the increased synthesis of cardiac angiotensins occurred only in patients with increased ESS.

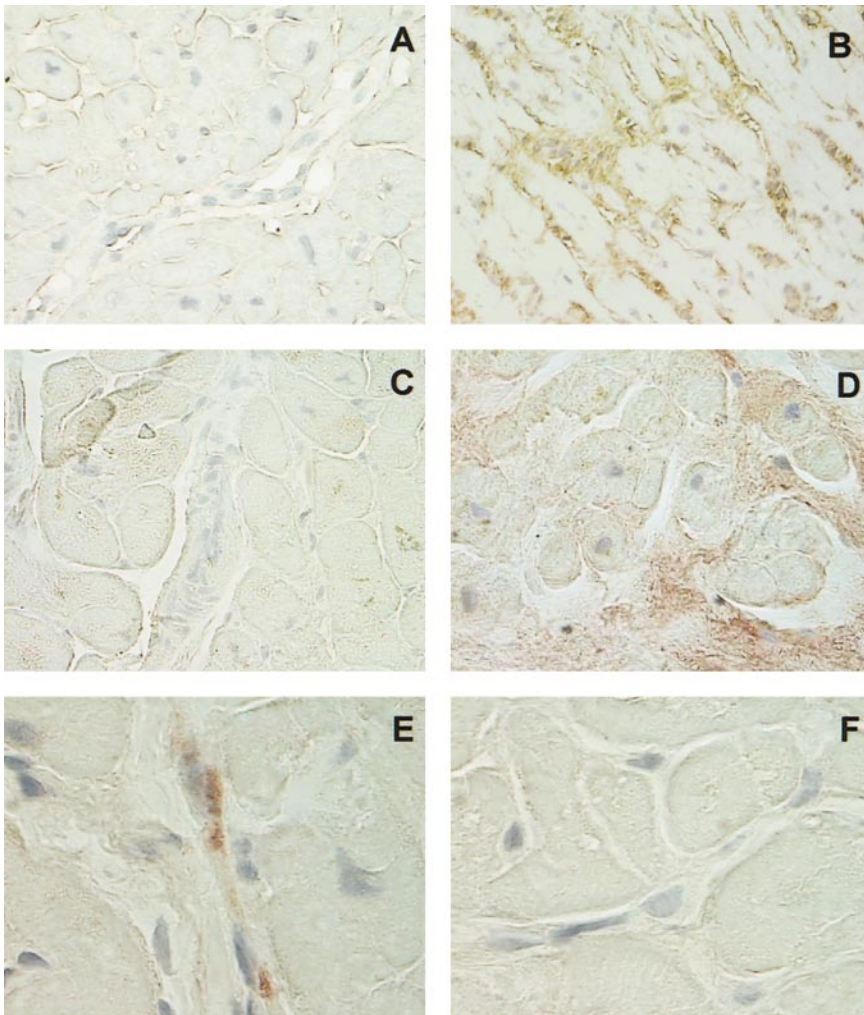
### Discussion

The present study shows that in humans, (1) left ventricular hypertrophy is associated with an increased cardiac formation of several growth factors, (2) growth factors are produced

selectively in relation to the type of hemodynamic overload, and (3) growth factor formation changes in relation to systolic wall stress.

### Compensatory Hypertrophy and Selective Formation of Growth Factors

Both the measurements of the active peptides in coronary sinus blood and the evaluation of mRNA for IGF-I and ppET-1 by RT-PCR showed that adequate or compensatory hypertrophy was associated with a selective increase in cardiac generation of IGF-I (enhanced in both AS and in AR) and ET-1 (increased only in AS). These findings suggest that an increase in IGF-I formation is a primary nonselective cardiac response to increased workload, whereas a more selective stimulus, such as pressure overload, is required to enhance ET-1 formation. Evidence has been provided both in vitro and in vivo that mechanical forces can selectively regulate gene expression and cause differential induction of peptide growth factors.<sup>27-29</sup> The augmented formation of IGF-I and ET-1 depends on the increased left ventricle load and is not related to possible derangements of pulmonary or peripheral hemodynamics, because no evidence of enhanced cardiac growth factor formation was found in patients with mitral stenosis despite the presence of atrial fibrillation,



**Figure 8.** In situ hybridization for AGTN mRNA in myocardial biopsies from patients with AR (A, B, and E) or AS (C and D) with adequate (A and C) and inadequate hypertrophy (B, D, and E). F, RNase treatment of the same specimen of panel E. Magnification:  $\times 400$  (A through D) and  $\times 1000$  (E and F). A, mRNA signal is almost undetectable in a myocardial specimen from a patient with AR and adequate hypertrophy. B and E, Signal of mRNA expression is evident in interstitial cells in AR with inadequate hypertrophy. C, Almost-undetectable signal of mRNA in AS with adequate hypertrophy. D, Markedly positive signal in interstitial cells of a patient with AS and inadequate hypertrophy.

enhanced pulmonary arterial pressure, and increased levels of circulating ET-1 and Big ET. Likewise, the occurrence of stable effort angina in the formation of ET-1 and IGF-I seems to play a minor role, given that no significant differences were found between patients with and patients without angina and coronary artery disease.

Hybridization studies showed that IGF-I in patients with AR was essentially synthesized by myocytes and its formation was closely associated with preserved ventricular contractility, because IGF-I formation was no longer detectable when Vcf was reduced. IGF-I directly induces hypertrophy in isolated cardiomyocytes<sup>30</sup> and enhances ventricular hypertrophy and myocyte function with no or only a mild increase in myocardial fibrosis in adult rats.<sup>31,32</sup> Constitutive overexpression of IGF-I in transgenic mice positively influences the performance of myocytes by enhancing the shortening velocity and cellular compliance, with consequent improvement of myocardial response to the Frank-Starling relation.<sup>33</sup> Thus, these properties of IGF-I fit very well with the functional and morphological characteristics of the hemodynamic compensation of AR, which is substantially a “magnification” type growth.<sup>16</sup> This is borne out by the elongation of sarcomeres with mild wall thickness, which results in increased left ventricular volume with a low ratio between myocardial

collagen fiber content and LVEDVI.<sup>34</sup> In AS with adequate hypertrophy, mRNA for IGF-I was mainly expressed by myocytes and mRNA for ppET-1 by both myocytes and, to a lesser extent, interstitial cells. In patients with AS, both ET-1 and IGF-I were positively correlated to RWT ( $r=0.82$  and  $r=0.68$ ) and Vcf ( $r=0.68$  and  $r=0.76$ ), which suggests a synergistic role of these peptides in supporting both the contractility and ventricular wall thickening needed to counterbalance the increased endoventricular systolic pressure.<sup>35</sup> Thus, compensatory hypertrophy, regardless of the type of overload, is sustained by the capacity of myocytes to generate growth factors endowed with inotropic activity, such as IGF-I, and, in pressure overload, factors that, besides the inotropic property, have the capacity for increasing wall thickness, such as ET-1.

#### Ang Formation and Transition to Heart Failure

The patients with ESS  $>90$  kdyn/cm<sup>2</sup> showed depressed ventricular contractility, as demonstrated by the low Vcf/LVEDVI ratio, and biochemically were characterized by a notable decrease in or even absent generation of IGF-I and ET-1 by myocytes and by the increase in Ang II generation. mRNA for these factors was almost exclusively expressed by interstitial cells. The reduced capacity of myocytes to synthe-



size IGF-I and ET-1 might be an aspect of phenotype changes related to the progressive severity of hypertrophy<sup>36–38</sup> and, most importantly, responsible for further depression of contractility. Alternatively, the increased wall stress might inhibit ET-1 and IGF-I generation and at the same time induce Ang II formation, given that the mechanical forces acting in different ways produce different effects on gene expression and protein synthesis.<sup>5,39</sup>

In patients with ESS >90 kdyne/cm<sup>2</sup>, Ang II was the main growth factor synthesized regardless of the type of hemodynamic overload, and the generation of Ang II was closely related to ESS, EDS, and LVEDP. The mRNA for AGTN was essentially expressed by the interstitial cells (Figure 8). Of course, the lower sensitivity of in situ hybridization in comparison with quantitative analysis does not rule out the possibility that mRNA for AGTN may also be expressed in cardiomyocytes. The high correlation between Ang II formation and wall stress suggests that ventricular distension is a causative factor for Ang II formation in humans, as in isolated myocytes<sup>40,41</sup> and beating hearts.<sup>42</sup>

Although Ang II has been found to induce myocyte hypertrophy<sup>4</sup> and increased Ang II formation has been frequently observed in experimental models of hypertrophy,<sup>43</sup> the hypertrophic response to hemodynamic overload is not inhibited by Ang blockade,<sup>44</sup> and its role in myocardial hypertrophy is still under debate.<sup>45</sup> In the present study, Ang II formation was not increased in patients with compensated hypertrophy and was positively correlated to the indices of reduced ventricular function and ventricular distension. On the whole, the present results indicate that Ang II is not a factor for myocardial hypertrophy in humans, but rather a growth factor that expresses cardiac maladaptation to the increased workload. The participation of Ang II in myocardial hypertrophy may be related mainly to the development of myocardial fibrosis, as suggested by several in vitro and experimental studies.<sup>46,47</sup> Moreover, the chronically increased Ang II formation may favor myocyte apoptosis.<sup>41,48</sup>

In conclusion, the present results indicate that the course of human myocardial hypertrophy is characterized by the participation of different growth factors related both to the type of hemodynamic overload and to the functional characteristics of the ventricle. The different hemodynamic overload leads to a selective formation of IGF-I (volume load) or of both IGF-I and ET-1 (pressure overload), and the ensuing increased wall stress brings Ang II formation into action. In addition to pathophysiological significance, these results may have important clinical implications, because serial echocardiographic measurements of ESS in asymptomatic patients with myocardial hypertrophy allow the timely administration of angiotensin-converting enzyme inhibitors or other Ang II antagonists that have been shown<sup>49,50</sup> to prevent or delay the development of contractile dysfunction and the transition to heart failure in rats and humans.

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